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TITLE OF THE INVENTION (500 characters max)

MAXs AS MODIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

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☐ Application Data Sheet. See 37 CFR 1.76

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☐ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

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REGISTRATION NO. 47,937
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PROVISIONAL APPLICATION COVER SHEET

Additional Page

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Docket Number	EX02-108P

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Number 1 of 1

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EX02-108P

MAXs AS MODIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

BACKGROUND OF THE INVENTION

Deregulation of ß-catenin signaling is a frequent and early event in the development of a variety of human tumors, including colon cancer, melanoma, ovarian cancer, and prostate cancer. Activation of ß -catenin signaling can occur in tumor cells by loss-of-function mutations in the tumor suppressor genes Axin or APC, as well as by gain-of-function mutations in the oncogene ß -catenin itself. Axin normally functions as a scaffolding protein that binds ß -catenin, APC, and the serine/threonine kinase GSK3 ß. Assembly of this degradation complex allows GSK3 ß to phosphorylate ß -catenin, which leads to ß -catenin ubiquitination and degradation by the proteasome. In the absence of Axin activity, ß -catenin protein becomes stabilized and accumulates in the nucleus where it acts as a transcriptional co-activator with TCF for the induction of target genes, including the cell cycle regulators cyclin D1 and c-Myc.

The *C. elegans* gene *pry-1* is the structural and functional ortholog of vertebrate Axin (Korswagen HC et al. (2002) Genes Dev. 16:1291-302). PRY-1 is predicted to contain conserved RGS and DIX domains that, in Axin, bind APC and Dishevelled, respectively. Overexpression of the *C. elegans pry-1* gene in zebrafish can fully rescue the mutant phenotype of *masterblind*, the zebrafish Axin1 mutation. *pry-1* loss-of-function mutations produce several phenotypes that appear to result from increased ß -catenin signaling (Gleason JE et al. (2002) Genes Dev. 16:1281-90; Korswagen et al., *supra*).

Membrane-bound transcription factor protease site 2(MBTPS2 or S2P) is a transmembrane metalloprotease required for intramembrane proteolysis of sterol regulatory element-binding proteins (SREBPs) at site 2 (Rawson, R. B. et al (1997) Molec. Cell 1: 47-57). SREBPs are membrane-bound transcription factors that activate genes regulating cholesterol metabolism. The active N-terminal domains of SREBPs are released from membranes by sequential cleavage at 2 sites: site 1, within the lumen of the endoplasmic reticulum (ER), and site 2, within a transmembrane segment. MBTPS2 is required for the ER stress response as well as for lipid synthesis (Ye, J. et al (2000) Molec. Cell 6: 1355-1364).

Three-prime-phosphoadenosine 5-prime-phosphosulfate (PAPS) is the sulfate donor cosubstrate for all sulfotransferase (SULT) enzymes. SULTs catalyze the sulfate conjugation of many endogenous and exogenous compounds, including drugs and other xenobiotics. In humans, PAPS is synthesized from adenosine 5-prime triphosphate (ATP) and inorganic sulfate by 2 isoforms, phosphoadenosine-phosphosulfate synthetase 1 (PAPSS1) and phosphoadenosine-phosphosulfate synthetase 2 (PAPSS2). PAPSS enzymes have kinase and sulfurylase domains, and catalyze 2 sequential reactions to synthesize PAPS. These reactions are catalyzed by separate enzymes encoded by 2 or 3 genes in simpler organisms. Mutations in PAPSS2 may result in spondyloepimetaphyseal dysplasia (ul Haque, M. F (1998) Nat Genet 20:157-62), and PAPSS2 is expressed in metastatic and non-metastatic colon carcinoma cells (Franzon, V. L. et al (1999) Int J Biochem Cell Biol 31:613-26).

PAPSS genes are conserved through evolution and have orthologs in yeast and higher eukaryotes, including a marine worm, Drosophila, and mouse (Yanagisawa, K. et al (1998) Biosci. Biotech. Biochem. 62: 1037-1040).

Chromosomal segregation during mitosis as well as meiosis is regulated by kinases and phosphatases. The Drosophila 'aurora' and S. cerevisiae Ipl1 serine/threonine protein kinases (STKs) are involved in mitotic events such as centrosome separation and chromosome segregation. Human STK6, STK 12, and STK13 are related to Drosophila 'aurora' and S. cerevisiae Ipl1 (Kimura, M. et al (1997) J. Biol. Chem. 272: 13766-13771; Katayama, H. et al (1998) Gene 224: 1-7; Kimura, M. et al. (1998) Cytogenet. Cell Genet. 82: 147-152; Bernard, M. et al. (1998) Genomics 53: 406-409, 1998).

STK6 is expressed in many tumor lines (Tanaka, T. et al. (1999) Cancer Res 59:2041-4). STK12 is highly expressed in transformed cell lines, and is associated with multinuclearity and polyploidy (Tatsuka, M. et al. (1998) Cancer Res. 58: 4811-4816).

The ability to manipulate the genomes of model organisms such as *C. elegans* provides a powerful means to analyze biochemical processes that, due to significant evolutionary conservation, have direct relevance to more complex vertebrate organisms. Due to a high level of gene and pathway conservation, the strong similarity of cellular processes, and the functional conservation of genes between these model organisms and mammals, identification of the involvement of novel

genes in particular pathways and their functions in such model organisms can directly contribute to the understanding of the correlative pathways and methods of modulating them in mammals (see, for example, Dulubova I, et al, J Neurochem 2001 Apr;77(1):229-38; Cai T, et al., Diabetologia 2001 Jan;44(1):81-8; Pasquinelli AE, et al., Nature. 2000 Nov 2;408(6808):37-8; Ivanov IP, et al., EMBO J 2000 Apr 17;19(8):1907-17; Vajo Z et al., Mamm Genome 1999 Oct;10(10):1000-4). For example, a genetic screen can be carried out in an invertebrate model organism having underexpression (e.g. knockout) or overexpression of a gene (referred to as a "genetic entry point") that yields a visible phenotype. Additional genes are mutated in a random or targeted manner. When a gene mutation changes the original phenotype caused by the mutation in the genetic entry point, the gene is identified as a "modifier" involved in the same or overlapping pathway as the genetic entry point. When the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as axin, modifier genes can be identified that may be attractive candidate targets for novel therapeutics.

All references cited herein, including sequence information in referenced Genbank identifier numbers and website references, are incorporated herein in their entireties.

SUMMARY OF THE INVENTION

We have discovered genes that modify the axin pathway in *C. elegans*, and identified their human orthologs, hereinafter referred to as MAX (Modifer of Axin). The invention provides methods for utilizing these axin modifier genes and polypeptides to identify MAX-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired axin function and/or MAX function. Preferred MAX-modulating agents specifically bind to MAX polypeptides and restore axin function. Other preferred MAX-modulating agents are nucleic acid modulators such as antisense oligomers and RNAi that repress MAX gene expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

MAX modulating agents may be evaluated by any convenient *in vitro* or *in vivo* assay for molecular interaction with a MAX polypeptide or nucleic acid. In one embodiment, candidate MAX modulating agents are tested with an assay system comprising a MAX polypeptide or nucleic acid. Agents that produce a change in the activity of the assay system relative to controls are identified as candidate axin modulating agents. The assay system may be cell-based or cell-free. MAX-modulating agents include MAX related proteins (e.g. dominant negative mutants, and biotherapeutics); MAX-specific antibodies; MAX-specific antisense oligomers and other nucleic acid modulators; and chemical agents that specifically bind to or interact with MAX or compete with MAX binding partner (e.g. by binding to a MAX binding partner). In one specific embodiment, a small molecule modulator is identified using a binding assay. In specific embodiments, the screening assay system is selected from an apoptosis assay, a cell proliferation assay, an angiogenesis assay, and a hypoxic induction assay.

In another embodiment, candidate axin pathway modulating agents are further tested using a second assay system that detects changes in the axin pathway, such as angiogenic, apoptotic, or cell proliferation changes produced by the originally identified candidate agent or an agent derived from the original agent. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating the axin pathway, such as an angiogenic, apoptotic, or cell proliferation disorder (e.g. cancer).

The invention further provides methods for modulating the MAX function and/or the axin pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a MAX polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to a mammalian animal predetermined to have a pathology associated the axin pathway.

DETAILED DESCRIPTION OF THE INVENTION

Genetic screens were designed to identify modifiers of the axin pathway in *C. elegans,* where a reduction of function pry-1 (axin) mutant was used. Various specific genes were silenced by RNA inhibition (RNAi). Methods for using RNAi to silence genes in *C. elegans* are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); WO9932619). Genes causing altered phenotypes in the worms were identified as modifiers of the axin pathway, followed by identification of their human orthologs (modifier of axin; MAX). Table 1 lists the modifiers and their orthologs.

In vitro and in vivo methods of assessing MAX function are provided herein. Modulation of the MAX or their respective binding partners is useful for understanding the association of the axin pathway and its members in normal and disease conditions and for developing diagnostics and therapeutic modalities for axin related pathologies. MAX-modulating agents that act by inhibiting or enhancing MAX expression, directly or indirectly, for example, by affecting a MAX function such as enzymatic (e.g., catalytic) or binding activity, can be identified using methods provided herein. MAX modulating agents are useful in diagnosis, therapy and pharmaceutical development.

<u>Nucleic acids and polypeptides of the invention</u>

Sequences related to MAX nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) number), shown in Table 1 and in the appended sequence listing .

Preferred MAXs are enzymes including protein kinases and proteases, among others.

The term "MAX polypeptide" refers to a full-length MAX protein or a functionally active fragment or derivative thereof. A "functionally active" MAX fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type MAX protein, such as antigenic or immunogenic activity, enzymatic activity, ability to bind natural cellular substrates, etc. The functional activity of MAX proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998)

Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey) and as further discussed below. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of a MAX, such as a kinase domain or a binding domain. Protein domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2). Methods for obtaining MAX polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of a MAX. In further preferred embodiments, the fragment comprises the entire functionally active domain.

The term "MAX nucleic acid" refers to a DNA or RNA molecule that encodes a MAX polypeptide. Preferably, the MAX polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with MAX. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994, Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. In

evolution, when a gene duplication event follows speciation, a single gene in one species, such as *C.elegans*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, Advances in Applied Mathematics 2:482-489; database: European Bioinformatics Institute;

Smith and Waterman, 1981, J. of Molec.Biol., 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.; W.R. Pearson, 1991, Genomics 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff (Dayhoff: Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 Nucl. Acids Res. 14(6):6745-6763). The Smith-Waterman algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated, the "Match" value reflects "sequence identity."

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of a MAX. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of a mAX under stringent hybridization conditions that comprise: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 μg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 μg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.2X SSC and 0.1% SDS (sodium dodecyl sulfate).

In other embodiments, moderately stringent hybridization conditions are used that comprise: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1%

PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Alternatively, low stringency conditions can be used that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

Isolation, Production, Expression, and Mis-expression of MAX Nucleic Acids and Polypeptides

MAX nucleic acids and polypeptides, useful for identifying and testing agents that modulate MAX function and for other applications related to the involvement of MAX in the axin pathway. MAX nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods. For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of a MAX protein for assays used to assess MAX function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (e.g., Higgins SJ and Hames BD (eds.) Protein Expression: A Practical Approach, Oxford University Press

Inc., New York 1999; Stanbury PF et al., Principles of Fermentation Technology, 2nd edition, Elsevier Science, New York, 1995; Doonan S (ed.) Protein Purification Protocols, Humana Press, New Jersey, 1996; Coligan JE et al, Current Protocols in Protein Science (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant MAX is expressed in a cell line known to have defective axin function. The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

The nucleotide sequence encoding a MAX polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native MAX gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. A host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

To detect expression of the MAX gene product, the expression vector can comprise a promoter operably linked to a MAX gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the MAX gene product based on the physical or functional properties of the MAX protein in *in vitro* assay systems (*e.g.* immunoassays).

The MAX protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (i.e. it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. A chimeric product may also

be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

Once a recombinant cell that expresses the MAX gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). Alternatively, native MAX proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of MAX or other genes associated with the axin pathway. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

Genetically modified animals

Animal models that have been genetically modified to alter MAX expression may be used in *in vivo* assays to test for activity of a candidate axin modulating agent, or to further assess the role of MAX in a axin pathway process such as apoptosis or cell proliferation. Preferably, the altered MAX expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal MAX expression. The genetically modified animal may additionally have altered axin expression (e.g. axin knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice), cows, horses, goats, sheep, pigs, dogs and cats. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4:761-763.) or stably integrated into its germ line DNA (i.e., in the genomic

sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al., and Hogan, B., Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sandford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, Science (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et al.*, A Universal Marker for Transgenic Insects (1999) Nature 402:370-371; for transgenic Zebrafish see Lin S., Transgenic Zebrafish, Methods Mol Biol. (2000);136:375-3830); for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, Experientia (1991) 47:897-905; for transgenic rats see Hammer *et al.*, Cell (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) Nature 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous MAX gene that results in a decrease of MAX function, preferably such that MAX expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a

mouse MAX gene is used to construct a homologous recombination vector suitable for altering an endogenous MAX gene in the mouse genome. Detailed methodologies for homologous recombination in mice are available (see Capecchi, Science (1989) 244:1288-1292; Joyner *et al.*, Nature (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, Science (1989) 244:1281-1288; Simms *et al.*, Bio/Technology (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene that has been knocked out (Claesson MH et al., (1994) Scan J Immunol 40:257-264; Declerck PJ et al., (1995) J Biol Chem. 270:8397-400).

In another embodiment, the transgenic animal is a "knock-in" animal having an alteration in its genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the MAX gene, e.g., by introduction of additional copies of MAX, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the MAX gene. Such regulatory sequences include inducible, tissue-specific, and constitutive promoters and enhancer elements. The knock-in can be homozygous or heterozygous.

Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*, PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of Saccharomyces cerevisiae (O'Gorman et al. (1991) Science 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the

transgene, and for sequential deletion of vector sequences in the same cell (Sun X et al (2000) Nat Genet 25:83-6).

The genetically modified animals can be used in genetic studies to further elucidate the axin pathway, as animal models of disease and disorders implicating defective axin function, and for *in vivo* testing of candidate therapeutic agents, such as those identified in screens described below. The candidate therapeutic agents are administered to a genetically modified animal having altered MAX function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that receive placebo treatment, and/or animals with unaltered MAX expression that receive candidate therapeutic agent.

In addition to the above-described genetically modified animals having altered MAX function, animal models having defective axin function (and otherwise normal MAX function), can be used in the methods of the present invention. For example, a axin knockout mouse can be used to assess, *in vivo*, the activity of a candidate axin modulating agent identified in one of the *in vitro* assays described below. Preferably, the candidate axin modulating agent when administered to a model system with cells defective in axin function, produces a detectable phenotypic change in the model system indicating that the axin function is restored, i.e., the cells exhibit normal cell cycle progression.

<u>Modulating Agents</u>

The invention provides methods to identify agents that interact with and/or modulate the function of MAX and/or the axin pathway. Modulating agents identified by the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the axin pathway, as well as in further analysis of the MAX protein and its contribution to the axin pathway. Accordingly, the invention also provides methods for modulating the axin pathway comprising the step of specifically modulating MAX activity by administering a MAX-interacting or -modulating agent.

As used herein, an "MAX-modulating agent" is any agent that modulated MAX function, for example, an agent that interacts with MAX to inhibit or enhance MAX

activity or otherwise affect normal MAX function. MAX function can be affected at any level, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a preferred embodiment, the MAX - modulating agent specifically modulates the function of the MAX. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the MAX polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the MAX. These phrases also encompasses modulating agents that alter the interaction of the MAX with a binding partner, substrate, or cofactor (e.g. by binding to a binding partner of a MAX, or to a protein/binding partner complex, and altering MAX function). In a further preferred embodiment, the MAX- modulating agent is a modulator of the axin pathway (e.g. it restores and/or upregulates axin function) and thus is also a axin-modulating agent.

Preferred MAX-modulating agents include small molecule compounds; MAX-interacting proteins, including antibodies and other biotherapeutics; and nucleic acid modulators such as antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

Small molecule modulators

Small molecules, are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more preferably less than 1,000, and most preferably less than 500. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the MAX protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are natural products,

particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for MAX–modulating activity. Methods for generating and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the axin pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

Protein Modulators

Specific MAX-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to the axin pathway and related disorders, as well as in validation assays for other MAX-modulating agents. In a preferred embodiment, MAX-interacting proteins affect normal MAX function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, MAX-interacting proteins are useful in detecting and providing information about the function of MAX proteins, as is relevant to axin related disorders, such as cancer (e.g., for diagnostic means).

An MAX-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with a MAX, such as a member of the MAX pathway that modulates MAX expression, localization, and/or activity. MAX-modulators include dominant negative forms of MAX-interacting proteins and of MAX proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous MAX-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression

Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3rd, Trends Genet (2000) 16:5-8).

An MAX-interacting protein may be an exogenous protein, such as a MAX-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory; Harlow and Lane (1999) Using antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). MAX antibodies are further discussed below.

In preferred embodiments, a MAX-interacting protein specifically binds a MAX protein. In alternative preferred embodiments, a MAX-modulating agent binds a MAX substrate, binding partner, or cofactor.

Antibodies

In another embodiment, the protein modulator is a MAX specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify MAX modulators. The antibodies can also be used in dissecting the portions of the MAX pathway responsible for various cellular responses and in the general processing and maturation of the MAX.

Antibodies that specifically bind MAX polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of MAX polypeptide, and more preferably, to human MAX. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab').sub.2 fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Epitopes of MAX which are particularly antigenic can be selected, for example, by routine screening of MAX polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Nati. Acad. Sci. U.S.A. 78:3824-28; Hopp and Wood, (1983) Mol. Immunol. 20:483-89;

Sutcliffe et al., (1983) Science 219:660-66) to the amino acid sequence of a MAX. Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 M^{-1} to 10^{10} M^{-1}, or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of MAX or substantially purified fragments thereof. If MAX fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of a MAX protein. In a particular embodiment, MAX-specific antigens and/or immunogens are coupled to carrier proteins that stimulate the immune response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

The presence of MAX-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding MAX polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

Chimeric antibodies specific to MAX polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or

eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat. Nos. 5,530,101, 5,585,089, 5,693,762, and 6,180,370).

MAX-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No. 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989) 4:131-134). A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg –to

about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml to about10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

Nucleic Acid Modulators

Other preferred MAX-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit MAX activity. Preferred nucleic acid modulators interfere with the function of the MAX nucleic acid such as DNA replication, transcription, translocation of the MAX RNA to the site of protein translation, translation of protein from the MAX RNA, splicing of the MAX RNA to yield one or more mRNA species, or catalytic activity which may be engaged in or facilitated by the MAX RNA.

In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to a MAX mRNA to bind to and prevent translation, preferably by binding to the 5′ untranslated region. MAX-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that

facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

In another embodiment, the antisense oligomer is a phosphothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits, each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphodiamidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. :7:187-95; US Pat. No. 5,235,033; and US Pat No. 5,378,841).

Alternative preferred MAX nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498).

Nucleic acid modulators are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Nucleic acid modulators are also used, for example, to distinguish between functions of various members of a biological pathway. For example, antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in

numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, a MAX-specific nucleic acid modulator is used in an assay to further elucidate the role of the MAX in the axin pathway, and/or its relationship to other members of the pathway. In another aspect of the invention, a MAX-specific antisense oligomer is used as a therapeutic agent for treatment of axin-related disease states.

Assay Systems

The invention provides assay systems and screening methods for identifying specific modulators of MAX activity. As used herein, an "assay system" encompasses all the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the MAX nucleic acid or protein. In general, secondary assays further assess the activity of a MAX modulating agent identified by a primary assay and may confirm that the modulating agent affects MAX in a manner relevant to the axin pathway. In some cases, MAX modulators will be directly tested in a secondary assay.

In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising a MAX polypeptide or nucleic acid with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. kinase activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates MAX activity, and hence the axin pathway. The MAX polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

Primary Assays

The type of modulator tested generally determines the type of primary assay.

Primary assays for small molecule modulators

For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding), transcriptional activity (*e.g.*, using a reporter gene), enzymatic activity (*e.g.*, via a property of the substrate), activity of second messengers, immunogenicty and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular molecular event detected.

Cell-based screening assays usually require systems for recombinant expression of MAX and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when MAX-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the MAX protein may be assayed by various known methods such as substrate processing (e.g. ability of the candidate MAX-specific binding agents to function as negative effectors in MAX-expressing cells), binding equilibrium constants (usually at least about 10^7 M^{-1}, preferably at least about 10^8 M^{-1}, more preferably at least about 10^9 M^{-1}), and immunogenicity (e.g. ability to elicit MAX specific antibody in a

heterologous host such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of a MAX polypeptide, a fusion protein thereof, or to cells or membranes bearing the polypeptide or fusion protein. The MAX polypeptide can be full length or a fragment thereof that retains functional MAX activity. The MAX polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The MAX polypeptide is preferably human MAX, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay detects candidate agent-based modulation of MAX interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has MAX –specific binding activity, and can be used to assess normal MAX gene function.

Suitable assay formats that may be adapted to screen for MAX modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, Curr Opin Chem Biol (1998) 2:597-603; Sundberg SA, Curr Opin Biotechnol 2000, 11:47-53). In one preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, Nat Struct Biol (2000) 7:730-4; Fernandes PB, *supra*; Hertzberg RP and Pope AJ, Curr Opin Chem Biol (2000) 4:445-451).

A variety of suitable assay systems may be used to identify candidate MAX and axin pathway modulators (e.g. U.S. Pat. No. 6,165,992 (kinase assays); U.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); U.S. Pat. No. 6,114,132 (phosphatase and protease assays), among others). Specific preferred assays are described in more detail below.

As seen from Table 1, preferred MAXs are enzymes including protein kinases and proteases, among others.

Proteases are enzymes that cleave protein substrates at specific sites. Exemplary assays detect the alterations in the spectral properties of an artificial substrate that occur upon protease-mediated cleavage. In one example, synthetic caspase substrates containing four amino acid proteolysis recognition sequences, separating two different fluorescent tags are employed; fluorescence resonance energy transfer detects the proximity of these fluorophores, which indicates whether the substrate is cleaved (Mahajan NP *et al.*, Chem Biol (1999) 6:401-409).

Protein kinases, key signal transduction proteins that may be either membrane-associated or intracellular, catalyze the transfer of gamma phosphate from adenosine triphosphate (ATP) to a serine, threonine or tyrosine residue in a protein substrate. In some preferred embodiments the screening assay detects the ability of the test agent to modulate the kinase activity of a MAX polypeptide. In further embodiments, a cell-free kinase assay system is used to identify a candidate axin modulating agent, and a secondary, cell-based assay, such as an apoptosis or hypoxic induction assay (described below), may be used to further characterize the candidate axin modulating agent. Many different assays for kinases have been reported in the literature and are well known to those skilled in the art (e.g. U.S. Pat. No. 6,165,992; Zhu et al., Nature Genetics (2000) 26:283-289; and WO0073469). Radioassays, which monitor the transfer of a gamma phosphate are frequently used. For instance, a scintillation assay for p56 (lck) kinase activity monitors the transfer of the gamma phosphate from gamma -^{33}P ATP to a biotinylated peptide substrate; the substrate is captured on a streptavidin coated bead that transmits the signal (Beveridge M *et al.*, J Biomol Screen (2000) 5:205-212). This assay uses the scintillation proximity assay (SPA), in which only radio-ligand bound to receptors tethered to the surface of an SPA bead are detected by the scintillant immobilized within it, allowing binding to be measured without separation of bound from free ligand.

Other assays for protein kinase activity may use antibodies that specifically recognize phosphorylated substrates. For instance, the kinase receptor activation (KIRA) assay measures receptor tyrosine kinase activity by ligand stimulating the intact receptor in cultured cells, then capturing solubilized receptor with specific antibodies and

quantifying phosphorylation via phosphotyrosine ELISA (Sadick MD, Dev Biol Stand (1999) 97:121-133).

Another example of antibody based assays for protein kinase activity is TRF (time-resolved fluorometry). This method utilizes europium chelate-labeled anti-phosphotyrosine antibodies to detect phosphate transfer to a polymeric substrate coated onto microtiter plate wells. The amount of phosphorylation is then detected using time-resolved, dissociation-enhanced fluorescence (Braunwalder AF, et al., Anal Biochem 1996 Jul 1;238(2):159-64).

Apoptosis assays. Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., et al., 1998, Blood 15:4730-41). An apoptosis assay system may comprise a cell that expresses a MAX, and that optionally has defective axin function (e.g. axin is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate axin modulating agents. In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate axin modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether MAX function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the MAX plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

Cell proliferation and cell cycle assays. Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA.

Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, Int. J. Cancer 38, 369; Campana *et al.*, 1988, J. Immunol. Meth. 107, 79), or by other means.

Cell Proliferation may also be examined using [^{3}H]-thymidine incorporation (Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [^{3}H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter).

Cell proliferation may also be assayed by colony formation in soft agar (Sambrook et al., Molecular Cloning, Cold Spring Harbor (1989)). For example, cells transformed with MAX are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray JW et al. (1986) Int J Radiat Biol Relat Stud Phys Chem Med 49:237-55). Cells transfected with a MAX may be stained with propidium iodide and evaluated in a flow cytometer (available from Becton Dickinson).

Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses a MAX, and that optionally has defective axin function (e.g. axin is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no test agent is added, identify candidate axin modulating agents. In some embodiments of the invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate axin modulating agents that is initially identified using another assay system such as a cell-free kinase assay system. A cell proliferation assay may also be used to test whether MAX function plays a direct role in cell proliferation or cell cycle. For example, a cell proliferation or cell cycle assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the MAX plays a direct role in cell proliferation or cell cycle.

Angiogenesis. Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses a MAX, and that optionally has defective axin function (e.g. axin is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate axin modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate axin modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether MAX function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the MAX plays a direct role in angiogenesis.

Hypoxic induction. The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glyolytic enzymes and VEGF. Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with MAX in hypoxic conditions (such as with 0.1% O2, 5% CO2, and balance N2, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses a MAX, and that optionally has a mutated axin (e.g. axin is over-expressed or under-expressed

relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate axin modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate axin modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether MAX function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in hypoxic response compared to wild type cells suggests that the MAX plays a direct role in hypoxic induction.

Cell adhesion. Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents. Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2x final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF , and allowed to adhere to the monolayers in the presence of candidate agents. Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., Bioconjug Chem. 2001 May-Jun;12(3):346-53).

Primary assays for antibody modulators

For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the MAX protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for detecting MAX-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

Primary assays for nucleic acid modulators

For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance MAX gene expression, preferably mRNA expression. In general, expression analysis comprises comparing MAX expression in like populations of cells (*e.g.*, two pools of cells that endogenously or recombinantly express MAX) in the presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that MAX mRNA expression is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, *eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with specific antibodies or antisera directed against either the MAX protein or

specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

Secondary Assays

Secondary assays may be used to further assess the activity of MAX-modulating agent identified by any of the above methods to confirm that the modulating agent affects MAX in a manner relevant to the axin pathway. As used herein, MAX-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with MAX.

Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express MAX) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate MAX–modulating agent results in changes in the axin pathway in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the axin or interacting pathways.

Cell-based assays

Cell based assays may use a variety of mammalian cell lines known to have defective axin function. Cell based assays may detect endogenous axin pathway activity or may rely on recombinant expression of axin pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

Animal Assays

A variety of non-human animal models of normal or defective axin pathway may be used to test candidate MAX modulators. Models for defective axin pathway typically use

genetically modified animals that have been engineered to mis-express (*e.g.*, over-express or lack expression in) genes involved in the axin pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

In a preferred embodiment, axin pathway activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal axin are used to test the candidate modulator's affect on MAX in Matrigel® assays. Matrigel® is an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4° C, but rapidly forms a solid gel at 37° C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells which over-express the MAX. The mixture is then injected subcutaneously(SC) into female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral (PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit). Hemoglobin content of the gel is found to correlate the degree of neovascularization in the gel.

In another preferred embodiment, the effect of the candidate modulator on MAX is assessed via tumorigenicity assays. In one example, xenograft human tumors are implanted SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the MAX endogenously are injected in the flank, 1×10^5 to 1×10^7 cells per mouse in a volume of 100 □L using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100 mg. Candidate modulator is delivered IV, SC, IP, or PO by bolus administration. Depending upon the pharmacokinetics of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors maybe utilized for biomarker identification or further

analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

Diagnostic and therapeutic uses

Specific MAX-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the axin pathway, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating the axin pathway in a cell, preferably a cell pre-determined to have defective or impaired axin function (e.g. due to overexpression, underexpression, or misexpression of axin, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates MAX activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the axin function is restored. The phrase "function is restored", and equivalents, as used herein, means that the desired phenotype is achieved, or is brought closer to normal compared to untreated cells. For example, with restored axin function, cell proliferation and/or progression through cell cycle may normalize, or be brought closer to normal relative to untreated cells. The invention also provides methods for treating disorders or disease associated with impaired axin function by administering a therapeutically effective amount of a MAX -modulating agent that modulates the axin pathway. The invention further provides methods for modulating MAX function in a cell, preferably a cell pre-determined to have defective or impaired MAX function, by administering a MAX -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired MAX function by administering a therapeutically effective amount of a MAX -modulating agent.

The discovery that MAX is implicated in axin pathway provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in the axin pathway and for the identification of subjects having a predisposition to such diseases and disorders.

Various expression analysis methods can be used to diagnose whether MAX expression occurs in a particular sample, including Northern blotting, slot blotting,

ribonuclease protection, quantitative RT-PCR, and microarray analysis. (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al., eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-47). Tissues having a disease or disorder implicating defective axin signaling that express a MAX, are identified as amenable to treatment with a MAX modulating agent. In a preferred application, the axin defective tissue overexpresses a MAX relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial MAX cDNA sequences as probes, can determine whether particular tumors express or overexpress MAX. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of MAX expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

Various other diagnostic methods may be performed, for example, utilizing reagents such as the MAX oligonucleotides, and antibodies directed against a MAX, as described above for: (1) the detection of the presence of MAX gene mutations, or the detection of either over- or under-expression of MAX mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of MAX gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by MAX.

Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease or disorder in a patient that is associated with alterations in MAX expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for MAX expression; c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of the disease or disorder. Preferably, the disease is cancer. The probe may be either DNA or protein, including an antibody.

EXAMPLES

The following experimental section and examples are offered by way of illustration and not by way of limitation.

I. C. elegans axin screen

We have found that the temperature-sensitive, reduction-of-function *pry-1* mutant *mu38* grown at 15°C produces a ruptured vulva (Rvl) phenotype by which about 95% of animals become eviscerated and die at the L4 molt. The *pry-1* Rvl mutant phenotype is suppressed by loss-of-function mutations in the ß -catenin ortholog *bar-1* and the TCF ortholog *pop-1*. The Rvl phenotype can also be generated by gain-of-function mutations in *bar-1*/ ß -catenin that eliminate the consensus GSK3 ß phosphorylation sites and are predicted to prevent Axin-mediated degradation of BAR-1.

We have designed a genetic screen to identify genes in addition to *bar-1*/ ß - catenin and *pop-1*/TCF that act positively in ß -catenin signaling and, when inactivated, suppress the Rvl mutant phenotype of *pry-1*/Axin. The function of individual genes is inactivated by RNAi in *pry-1 (mu38)* L1 larvae, and suppression of the Rvl phenotype is scored as a statistically significant increase in the proportion of larvae that survive to adulthood without rupturing. Suppressor genes are subsequently counterscreened to eliminate those that appear to suppress the *pry-1* mutant non-specifically, rather than those that specifically function in ß -catenin signaling. Suppressor genes that do not block vulva formation in a wildtype background, and that do not suppress the Rvl phenotype of two mutations in genes unrelated to ß -catenin signaling (*lin-1*/Ets and *daf-18*/PTEN) are considered to be specific *pry-1*/Axin suppressors. These suppressor genes, when inactivated, likely suppress ß -catenin's inappropriate transcriptional activation of target genes and, therefore, may be relevant for cancer therapy.

II. Analysis of Table 1

BLAST analysis (Altschul et al., *supra*) was employed to identify Targets from *C. elegans* modifiers. The columns "MAX symbol", and "MAX name aliases " provide a symbol and the known name abbreviations for the Targets, where available, from Genbank. "MAX RefSeq_NA or GI_NA", "MAX GI_AA", "MAX NAME", and "MAX Description" provide the reference DNA sequences for the MAXs as available from National Center for Biology Information (NCBI), MAX protein Genbank identifier

number (GI#), MAX name, and MAX description, all available from Genbank, respectively. The length of each amino acid is in the "MAX Protein Length" column.

Protein sequences of *C. elegans* modifiers of axin from screen (Example I), are represented in the "Modifier GI_AA" column by GI#.

III. High-Throughput In Vitro Fluorescence Polarization Assay

Fluorescently-labeled MAX peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of MAX activity.

IV. High-Throughput In Vitro Binding Assay.

^{33}P-labeled MAX peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM $MgCl_2$, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate axin modulating agents.

V. Immunoprecipitations and Immunoblotting

For coprecipitation of transfected proteins, 3×10^6 appropriate recombinant cells containing the MAX proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors

(complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at 15,000 × g for 15 min. The cell lysate is incubated with 25 μl of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

VI. Kinase assay

A purified or partially purified MAX is diluted in a suitable reaction buffer, e.g., 50 mM Hepes, pH 7.5, containing magnesium chloride or manganese chloride (1-20 mM) and a peptide or polypeptide substrate, such as myelin basic protein or casein (1-10 μg/ml). The final concentration of the kinase is 1-20 nM. The enzyme reaction is conducted in microtiter plates to facilitate optimization of reaction conditions by increasing assay throughput. A 96-well microtiter plate is employed using a final volume 30-100 μl. The reaction is initiated by the addition of ^{33}P-gamma-ATP (0.5 μCi/ml) and incubated for 0.5 to 3 hours at room temperature. Negative controls are provided by the addition of EDTA, which chelates the divalent cation ($Mg2^{+}$ or Mn^{2+}) required for enzymatic activity. Following the incubation, the enzyme reaction is quenched using EDTA. Samples of the reaction are transferred to a 96-well glass fiber filter plate (MultiScreen, Millipore). The filters are subsequently washed with phosphate-buffered saline, dilute phosphoric acid (0.5%) or other suitable medium to remove excess radiolabeled ATP. Scintillation cocktail is added to the filter plate and the incorporated radioactivity is quantitated by scintillation counting (Wallac/Perkin Elmer). Activity is defined by the amount of radioactivity detected following subtraction of the negative control reaction value (EDTA quench).

VII. Expression analysis

All cell lines used in the following experiments are NCI (National Cancer Institute) lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues are obtained from Impath, UC Davis, Clontech, Stratagene, and Ambion.

TaqMan analysis is used to assess expression levels of the disclosed genes in various samples.

RNA is extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/μl. Single stranded cDNA is then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of Applied Biosystems (Foster City, CA).

Primers for expression analysis using TaqMan assay (Applied Biosystems, Foster City, CA) are prepared according to the TaqMan protocols, and the following criteria: a) primer pairs are designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product.

Taqman reactions are carried out following manufacturer's protocols, in 25 μl total volume for 96-well plates and 10 μl total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis is prepared using a universal pool of human cDNA samples, which is a mixture of cDNAs from a wide variety of tissues so that the chance that a target will be present in appreciable amounts is good. The raw data are normalized using 18S rRNA (universally expressed in all tissues and cells).

For each expression analysis, tumor tissue samples are compared with matched normal tissues from the same patient. A gene is considered overexpressed in a tumor when the level of expression of the gene is 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue is not available, a universal pool of cDNA samples is used instead. In these cases, a gene is considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the average of all normal samples from the same tissue type is greater than 2 times the standard deviation of all normal samples (i.e., Tumor – average(all normal samples) > 2 x STDEV(all normal samples)).

A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression analysis as described above, by antibody directed to the gene target, or by any other available detection method.

WHAT IS CLAIMED IS:

1. A method of identifying a candidate axin pathway modulating agent, said method comprising the steps of:

(a) providing an assay system comprising a purified MAX polypeptide or nucleic acid or a functionally active fragment or derivative thereof;

(b) contacting the assay system with a test agent under conditions whereby, but for the presence of the test agent, the system provides a reference activity; and

(c) detecting a test agent-biased activity of the assay system, wherein a difference between the test agent-biased activity and the reference activity identifies the test agent as a candidate axin pathway modulating agent.

2. The method of Claim 1 wherein the assay system comprises cultured cells that express the MAX polypeptide.

3. The method of Claim 2 wherein the cultured cells additionally have defective axin function.

4. The method of Claim 1 wherein the assay system includes a screening assay comprising a MAX polypeptide, and the candidate test agent is a small molecule modulator.

5. The method of Claim 4 wherein the assay is a binding assay.

6. The method of Claim 1 wherein the assay system is selected from the group consisting of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay system, and a hypoxic induction assay system.

7. The method of Claim 1 wherein the assay system includes a binding assay comprising a MAX polypeptide and the candidate test agent is an antibody.

8. The method of Claim 1 wherein the assay system includes an expression assay comprising a MAX nucleic acid and the candidate test agent is a nucleic acid modulator.

9. The method of claim 8 wherein the nucleic acid modulator is an antisense oligomer.

10. The method of Claim 8 wherein the nucleic acid modulator is a PMO.

11. The method of Claim 1 additionally comprising:

(d) administering the candidate axin pathway modulating agent identified in (c) to a model system comprising cells defective in axin function and, detecting a phenotypic change in the model system that indicates that the axin function is restored.

12. The method of Claim 11 wherein the model system is a mouse model with defective axin function.

13. A method for modulating a axin pathway of a cell comprising contacting a cell defective in axin function with a candidate modulator that specifically binds to a MAX polypeptide, whereby axin function is restored.

14. The method of claim 13 wherein the candidate modulator is administered to a vertebrate animal predetermined to have a disease or disorder resulting from a defect in axin function.

15. The method of Claim 13 wherein the candidate modulator is selected from the group consisting of an antibody and a small molecule.

16. The method of Claim 1, comprising the additional steps of:

(d) providing a secondary assay system comprising cultured cells or a non-human animal expressing MAX ,

(e) contacting the secondary assay system with the test agent of (b) or an agent derived therefrom under conditions whereby, but for the presence of the test agent or agent derived therefrom, the system provides a reference activity; and

(f) detecting an agent-biased activity of the second assay system,

wherein a difference between the agent-biased activity and the reference activity of the second assay system confirms the test agent or agent derived therefrom as a candidate axin pathway modulating agent,

and wherein the second assay detects an agent-biased change in the axin pathway.

17. The method of Claim 16 wherein the secondary assay system comprises cultured cells.

18. The method of Claim 16 wherein the secondary assay system comprises a non-human animal.

19. The method of Claim 18 wherein the non-human animal mis-expresses a axin pathway gene.

20. A method of modulating axin pathway in a mammalian cell comprising contacting the cell with an agent that specifically binds a MAX polypeptide or nucleic acid.

21. The method of Claim 20 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with the axin pathway.

22. The method of Claim 20 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.

23. A method for diagnosing a disease in a patient comprising:

(a) obtaining a biological sample from the patient;

(b) contacting the sample with a probe for MAX expression;

(c) comparing results from step (b) with a control;

(d) determining whether step (c) indicates a likelihood of disease.

24. The method of claim 23 wherein said disease is cancer.

ABSTRACT OF THE DISCLOSURE

Human MAX genes are identified as modulators of the axin pathway, and thus are therapeutic targets for disorders associated with defective axin function. Methods for identifying modulators of axin, comprising screening for agents that modulate the activity of MAX are provided.

EX02-108P

Nucleic Acid and Polypeptide sequences

>gi|7706692|ref|NM_015884.1| Homo sapiens membrane-bound transcription factor protease, site 2 (MBTPS2), mRNA
AGCGGATGCTGGGGCTGTAAGGCGCGCGCGGTCAGCTGTTGGCGGTGCAGGGAGGAGGACGCCGGGGCTC
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ATAAAGTGT

>gi|20127474|ref|NM_005443.2| Homo sapiens 3'-phosphoadenosine 5'-phosphosulfate synthase 1 (PAPSS1), mRNA
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>gi|4758879|ref|NM_004670.1| Homo sapiens 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2), mRNA
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GTCTGGTCTGCATTACCAGCTTTATTTCTCCATTCGCAAAGGATCGTGAGAATGCCCGCAAAATACATGA
ATCAGCAGGGCTGCCATTCTTTGAAATATTTGTAGATGCACCTCTAAATATTTGTGAAAGCAGAGACGTA
AAAGGCCTCTATAAAAGGGCCAGAGCTGGGGAGATTAAAGGATTTACAGGTATTGATTCTGATTATGAGA
AACCTGAAACTCCTGAGCGTGTGCTTAAAACCAATTTGTCCACAGTGAGTGACTGTGTCCACCAGGTAGT
GGAACTTCTGCAAGAGCAGAACATTGTACCCTATACTATAATCAAAGATATCCACGAACTCTTTGTGCCG
GAAAACAAACTTGACCACGTCCGAGCTGAGGCTGAAACTCTCCCTTCATTATCAATTACTAAGCTGGATC
TCCAGTGGGTCCAGGTTTTGAGCGAAGGCTGGGCCACTCCCCTCAAAGGTTTCATGCGGGAGAAGGAGTA
CTTACAGGTTATGCACTTTGACACCCTGCTAGATGATGGCGTGATCAACATGAGCATCCCCATTGTACTG
CCCGTCTCTGCAGAGGATAAGACACGGCTGGAAGGGTGCAGCAAGTTTGTCCTGGCACATGGTGGACGGA
GGGTAGCTATCTTACGAGACGCTGAATTCTATGAACACAGAAAAGAGGAACGCTGTTCCCGTGTTTGGGG
GACAACATGTACAAAACACCCCCATATCAAAATGGTGATGGAAAGTGGGGACTGGCTGGTTGGTGGAGAC
CTTCAGGTGCTGGAGAAAATAAGATGGAATGATGGGCTGGACCAATACCGTCTGACACCTCTGGAGCTCA
AACAGAAATGTAAAGAAATGAATGCTGATGCGGTGTTTGCATTCCAGTTGCGCAATCCTGTCCACAATGG
CCATGCCCTGTTGATGCAGGACACCTGCCGCAGGCTCCTAGAGAGGGGCTACAAGCACCCGGTCCTCCTA
CTACACCCTCTGGGCGGCTGGACCAAGGATGACGATGTGCCTCTAGACTGGCGGATGAAGCAGCACGCGG
CTGTGCTCGAGGAAGGGGTCCTGGATCCCAAGTCAACCATTGTTGCCATCTTTCCGTCTCCCATGTTATA
TGCTGGCCCCACAGAGGTCCAGTGGCACTGCAGGTCCCGGATGATTGCGGGTGCCAATTTCTACATTGTG
GGGAGGGACCCTGCAGGAATGCCCCATCCTGAAACCAAGAAGGATCTGTATGAACCCACTCATGGGGGCA
AGGTCTTGAGCATGGCCCCTGGCCTCACCTCTGTGGAAATCATTCCATTCCGAGTGGCTGCCTACAACAA
AGCCAAAAAAGCCATGGACTTCTATGATCCAGCAAGGCACAATGAGTTTGACTTCATCTCAGGAACTCGA
ATGAGGAAGCTCGCCCGGGAAGGAGAGAATCCCCCAGATGGCTTCATGGCCCCCAAAGCATGGAAGGTCC
TGACAGATTATTACAGGTCCCTGGAGAAGAACTAAGCCTTTGGGTCCAGAGTTTCTTTCTGAAGTGCTCT
TTGATTACCTTTTCTATTTTTATGATTAGATGCTTTGTATTAAATTGCTTCTCA

>gi|4759177|ref|NM_004217.1| Homo sapiens serine/threonine kinase 12 (STK12), mRNA
GGCCGGGAGAGTAGCAGTGCCTTGGACCCCAGCTCTCCTCCCCCTTTCTCTCTAAGGATGGCCCAGAAGG
AGAACTCCTACCCCTGGCCCTACGGCCGACAGACGGCTCCATCTGGCCTGAGCACCCTGCCCCAGCGAGT
CCTCCGGAAAGAGCCTGTCACCCCATCTGCACTTGTCCTCATGAGCCGCTCCAATGTCCAGCCCACAGCT
GCCCCTGGCCAGAAGGTGATGGAGAATAGCAGTGGGACACCCGACATCTTAACGCGGCACTTCACAATTG
ATGACTTTGAGATTGGGCGTCCTCTGGGCAAAGGCAAGTTTGGAAACGTGTACTTGGCTCGGGAGAAGAA
AAGCCATTTCATCGTGGCGCTCAAGGTCCTCTTCAAGTCCCAGATAGAGAAGGAGGGCGTGGAGCATCAG
CTGCGCAGAGAGATCGAAATCCAGGCCCACCTGCACCATCCCAACATCCTGCGTCTCTACAACTATTTTT
ATGACCGGAGGAGGATCTACTTGATTCTAGAGTATGCCCCCCGCGGGGAGCTCTACAAGGAGCTGCAGAA
GAGCTGCACATTTGACGAGCAGCGAACAGCCACGATCATGGAGGAGTTGGCAGATGCTCTAATGTACTGC
CATGGGAAGAAGGTGATTCACAGAGACATAAAGCCAGAAAATCTGCTCTTAGGGCTCAAGGGAGAGCTGA
AGATTGCTGACTTCGGCTGGTCTGTGCATGCGCCCTCCCTGAGGAGGAAGACAATGTGTGGCACCCTGGA

CTACCTGCCCCCAGAGATGATTGAGGGGCGCATGCACAATGAGAAGGTGGATCTGTGGTGCATTGGAGTG
CTTTGCTATGAGCTGCTGGTGGGGAACCCACCCTTTGAGAGTGCATCACACAACGAGACCTATCGCCGCA
TCGTCAAGGTGGACCTAAAGTTCCCCGCTTCTGTGCCCACGGGAGCCCAGGACCTCATCTCCAAACTGCT
CAGGCATAACCCCTCGGAACGGCTGCCCCTGGCCCAGGTCTCAGCCCACCCTTGGGTCCGGGCCAACTCT
CGGAGGGTGCTGCCTCCCTCTGCCCTTCAATCTGTCGCCTGATGGTCCCTGTCATTCACTCGGGTGCGTG
TGTTTGTATGTCTGTGTATGTATAGGGGAAAGAAGGGATCCCTAACTGTTCCCTTATCTGTTTTCTACCT
CCTCCTTTGTTTAATAAAGGCTGAAGCTTTTTGT

>gi|4507272|ref|NM_003160.1| Homo sapiens serine/threonine kinase 13 (aurora/IPL1-like) (STK13), mRNA
CACAACGGCCATCACCACCTCTCCGCACCCTCTGTCTCCTTCCTTCTCGGGCCTCACCCCTGGCCCTCGC
TTTCAGTGCCCAGCCCGGACCGCAGCACTCCAGTTCCGCCCCAACCCGGGAACCCTGGACTGAGGCTCCC
GTTTCTGTCCTTTCTATTGGGCGCACTTCCGATGGCGTCAGGAATTTCAGCCAATAGGAGCCAGCCAGGA
AGTACCTCTCTGAGCGGTTGGTGCCGGGTATAAAAGAAGGCCAGCACGGCTGCTCACGACGCCGCGAAGC
CTGTGTAGCACTGAGACATCAGTGAGTTGGCTACAGCAGACCAAACAGCCCAGCAGCCCAGCAGCCCACG
CATGCGGCGCCTCACAGTCGATGACTTTGAAATCGGGCGTCCCCTGGGCAAGGGGAAATTTGGGAATGTG
TACCTGGCTCGGCTCAAGGAAAGCCATTTCATTGTGGCCCTGAAGGTTCTCTTCAAGTCGCAGATAGAGA
AGGAAGGACTGGAGCACCAGCTGCGCCGGGAAATTGAGATCCAGGCTCATCTACAACACCCCAATATCCT
GCGCCTGTATAACTATTTCCATGATGCACGCCGGGTGTACCTGATTCTGGAATATGCTCCAAGGGGTGAG
CTCTACAAGGAGCTGCAGAAAAGCGAGAAATTAGATGAACAGCGCACAGCCACGATAATAGAGGAAGTTG
CAGATGCCCTGACCTACTGCCATGACAAGAAAGTGATTCACAGAGATATTAAGCCAGAGAACCTGCTGCT
GGGGTTCAGGGGTGAGGTGAAGATTGCAGATTTTGGCTGGTCTGTGCACACCCCCCTCCCTGAGAGGAAG
ACAATGTGTGGGACACTGGACTACTTGCCGCCAGAAATGATTGAGGGGAGAACATATGATGAAAAGGTGG
ATTTGTGGTGCATTGGAGTGCTCTGCTATGAGCTGCTGGTGGGATATCCACCCTTTGAGAGCGCCTCCCA
CAGTGAGACTTACAGACGCATCCTCAAGGTAGATGTGAGGTTTCCACTATCAATGCCTCTGGGGGCCCGG
GACTTGATTTCCAGGCTTCTCAGATACCAGCCCTTGGAGAGACTGCCCCTGGCCCAGATCCTGAAGCACC
CCTGGGTTCAGGCCCACTCCCGAAGGGTGCTGCCTCCCTGTGCTCAGATGGCTTCCTGAGCCCTGTCTGC
CTCTGTTCCCTTTGTGTGTGTTCAGGGAGCTCTCCTGCTCTGCCACCTCATTTGTCTTTATTTTTTTCTC
TTTTAAGATGTAAGATGCTAATTAATAAAAGCTGAATCATTTCATACCAAAAAAAAAAAAAAAAAA

>gi|4507278|ref|NM_003158.1| Homo sapiens serine/threonine kinase 6 (STK6), mRNA
GAATTCCGGGACTGAGCTCTTGAAGACTTGGGTCCTTGGTCGCAGGTGGAGCGACGGGTCTCACTCCATT
GCCCAGGCCAGAGTGCGGGATATTTGATAAGAAACTTCAGTGAAGGCCGGGCGCGGTGCTCATGCCCGTA
ATCCCAGCATTTTCGGAGGCCGAGGCATCATGGACCGATCTAAAGAAAACTGCATTTCAGGACCTGTTAA
GGCTACAGCTCCAGTTGGAGGTCCAAAACGTGTTCTCGTGACTCAGCAATTTCCTTGTCAGAATCCATTA
CCTGTAAATAGTGGCCAGGCTCAGCGGGTCTTGTGTCCTTCAAATTCTTCCCAGCGCGTTCCTTTGCAAG
CACAAAAGCTTGTCTCCAGTCACAAGCCGGTTCAGAATCAGAAGCAGAAGCAATTGCAGGCAACCAGTGT
ACCTCATCCTGTCTCCAGGCCACTGAATAACACCCAAAAGAGCAAGCAGCCCCTGCCATCGCACCTGAAA
ATAATCCTGAGGAGGAACTGGCATCAAAACAGAAAAATGAAGAATCAAAAAGAGGCAGTGGCTTTGGAAG
ACTTTGAAATTGGTCGCCCTCTGGGTAAAGGAAAGTTTGGTAATGTTTATTTGGCAAGAGAAAAGCAAAG
CAAGTTTATTCTGGCTCTTAAAGTGTTATTTAAAGCTCAGCTGGAGAAAGCCGGAGTGGAGCATCAGCTC
AGAAGAGAAGTAGAAATACAGTCCCACCTTCGGCATCCTAATATTCTTAGACTGTATGGTTATTTCCATG
ATGCTACCAGAGTCTACCTAATTCTGGAATATGCACCACTTGGAACAGTTTATAGAGAACTTCAGAAACT
TTCAAAGTTTGATGAGCAGAGAACTGCTAACTTATATAACAGAATTGCAAATGCCCTGTCTTACTGTCAT
TCGAAGAGAGTTATTCATAGAGACATTAAGCCAGAGAACTTACTTCTTGGATCAGCTGGAGAGCTTAAAA
TTGCAGATTTTGGGTGGTCAGTACATGCTCCATCTTCCAGGAGGACCACTCTCTGTGGCACCCTGGACTA
CCTGCCCCCTGAAATGATTGAAGGTCGGATGCATGATGAGAAGGTGGATCTCTGGAGCCTTGGAGTTCTT
TGCTATGAATTTTTAGTTGGGAAGCCTCCTTTTGAGGCAAACACATACCAAGAGACCTACAAAAGAATAT
CACGGGTTGAATTCACATTCCCTGACTTTGTAACAGAGGGAGCCAGGGACCTCATTTCAAGACTGTTGAA
GCATAATCCCAGCCAGAGGCCAATGCTCAGAGAAGTACTTGAACACCCCTGGATCACAGCAAATTCATCA
AAACCATCAAATTGCCAAAACAAAGAATCAGCTAGCAAACAGTCTTAGGAATCGTGCAGGGGGAGAAATC
CTTGAGCCAGGGCTGCCATATAACCTGACAGGAACATGCTACTGAAGTTTATTTTACCATTGACTGCTGC
CCTCAATCTAGAACGCTACACAAGAAATATTTTGTTTTTACTCAGCAGGTGTGCCTTAACCTCCCTATTC
AGAAAGCTCCACATCAATAAACATGACACTCTGAAGTGAAAGTAGCCACGAGAATTGTGCTACTTATACT
GGAACATAATCTGGAGGCAAGGTTCGACTGCAGTCGAACCTTGCCTCCAGATTATGAACCAGTATAAGTA

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GCACAATTCTCGTGGCTACTTTCACTTCAGAGTGTCATGTTTATTGATGTGGAGCTTTCTGAATAGGGAG
GTTAAGGCACACCTGCTGAGTAAAACAAATATTTCTTGTGTAGCGTTCTTAGGAATCTGGTGTCTGTCCG
GCCCCGGTAGGCCTGTTGGGTTTCTAGTCCTCCTTACCATCATCTCCATATGAGAGTGTGAAAATAGGAA
CACGTGCTCTACCTCCATTTAGGGATTTGCTTGGGATACAGAAGAGGCCATGTGTCTCAGAGCTGTTAAG
GGCTTATTTTTTTAAAACATTGGAGTCATAGCATGTGTGTAAACTTTAAATATGCAGGCCTTCGTGGCTC
GAG

>gi|7706693|ref|NP_056968.1| membrane-bound transcription factor protease, site 2; S2P protein [Homo sapiens]
MIPVSLVVVVVGGWTVVYLTDLVLKSSVYFKHSYEDWLENNGLSISPFHIRWQTAVFNRAFYSWGRRKAR
MLYQWFNFGMVFGVIAMFSSFFLLGKTLMQTLAQMMADSPSSYSSSSSSSSSSSSSSSSSSSSSSLHNE
QVLQVVVPGINLPVNQLTYFFTAVLISGVVHEIGHGIAAIREQVRFNGFGIFLFIIYPGAFVDLFTTHLQ
LISPVQQLRIFCAGIWHNFVLALLGILALVLLPVILLPFYYTGVGVLITEVAEDSPAIGPRGLFVGDLVT
HLQDCPVTNVQDWNECLDTIAYEPQIGYCISASTLQQLSFPVRAYKRLDGSTECCNNHSLTDVCFSYRNN
FNKRLHTCLPARKAVEATQVCRTNKDCKKSSSSSFCIIPSLETHTRLIKVKHPPQIDMLYVGHPLHLHYT
VSITSFIPRFNFLSIDLPVVVETFVKYLISLSGALAIVNAVPCFALDGQWILNSFLDATLTSVIGDNDVK
DLIGFFILLGGSVLLAANVTLGLWMVTAR

>gi|20127475|ref|NP_005434.2| 3-prime-phosphoadenosine 5-prime-phosphosulfate synthase 1 [Homo sapiens]
MEIPGSLCKKVKLSNNAQNWGMQRATNVTYQAHHVSRNKRGQVVGTRGGFRGCTVWLTGLSGAGKTTVSM
ALEEYLVCHGIPCYTLDGDNIRQGLNKNLGFSPEDREENVRRIAEVAKLFADAGLVCITSFISPYTQDRN
NARQIHEGASLPFFEVFVDAPLHVCEQRDVKGLYKKARAGEIKGFTGIDSEYEKPEAPELVLKTDSCDVN
DCVQQVVELLQERDIVPVDASYEVKELYVPENKLHLAKTDAETLPALKINKVDMQWVQVLAEGWATPLNG
FMREREYLQCLHFDCLLDGGVINLSVPIVLTATHEDKERLDGCTAFALMYEGRRVAILRNPEFFEHRKEE
RCARQWGTTCKNHPYIKMVMEQGDWLIGGDLQVLDRVYWNDGLDQYRLTPTELKQKFKDMNADAVFAFQL
RNPVHNGHALLMQDTHKQLLERGYRRPVLLLHPLGGWTKDDDVPLMWRMKQHAAVLEEGVLNPETTVVAI
FPSPMMYAGPTEVQWHCRARMVAGANFYIVGRDPAGMPHPETGKDLYEPSHGAKVLTMAPGLITLEIVPF
RVAAYNKKKKRMDYYDSEHHEDFEFISGTRMRKLAREGQKPPEGFMAPKAWTVLTEYYKSLEKA

>gi|4758880|ref|NP_004661.1| 3-prime-phosphoadenosine 5-prime-phosphosulfate synthase 2 [Homo sapiens],
MSGIKKQKTENQQKSTNVVYQAHHVSRNKRGQVVGTRGGFRGCTVWLTGLSGAGKTTISFALEEYLVSHA
IPCYSLDGDNVRHGLNRNLGFSPGDREENIRRIAEVAKLFADAGLVCITSFISPFAKDRENARKIHESAG
LPFFEIFVDAPLNICESRDVKGLYKRARAGEIKGFTGIDSDYEKPETPERVLKTNLSTVSDCVHQVVELL
QEQNIVPYTIIKDIHELFVPENKLDHVRAEAETLPSLSITKLDLQWVQVLSEGWATPLKGFMREKEYLQV
MHFDTLLDDGVINMSIPIVLPVSAEDKTRLEGCSKFVLAHGGRRVAILRDAEFYEHRKEERCSRVWGTTC
TKHPHIKMVMESGDWLVGGDLQVLEKIRWNDGLDQYRLTPLELKQKCKEMNADAVFAFQLRNPVHNGHAL
LMQDTCRRLLERGYKHPVLLLHPLGGWTKDDDVPLDWRMKQHAAVLEEGVLDPKSTIVAIFPSPMLYAGP
TEVQWHCRSRMIAGANFYIVGRDPAGMPHPETKKDLYEPTHGGKVLSMAPGLTSVEIIPFRVAAYNKAKK
AMDFYDPARHNEFDFISGTRMRKLAREGENPPDGFMAPKAWKVLTDYYRSLEKN

>gi|4759178|ref|NP_004208.1| serine/threonine kinase 12 [Homo sapiens]
MAQKENSYPWPYGRQTAPSGLSTLPQRVLRKEPVTPSALVLMSRSNVQPTAAPGQKVMENSSGTPDILTR
HFTIDDFEIGRPLGKGKFGNVYLAREKKSHFIVALKVLFKSQIEKEGVEHQLRREIEIQAHLHHPNILRL
YNYFYDRRRIYLILEYAPRGELYKELQKSCTFDEQRTATIMEELADALMYCHGKKVIHRDIKPENLLLGL
KGELKIADFGWSVHAPSLRRKTMCGTLDYLPPEMIEGRMHNEKVDLWCIGVLCYELLVGNPPFESASHNE
TYRRIVKVDLKFPASVPTGAQDLISKLLRHNPSERLPLAQVSAHPWVRANSRRVLPPSALQSVA

>gi|4507273|ref|NP_003151.1| serine/threonine kinase 13 (aurora/IPL1-like) [Homo sapiens]
MRRLTVDDFEIGRPLGKGKFGNVYLARLKESHFIVALKVLFKSQIEKEGLEHQLRREIEIQAHLQHPNIL
RLYNYFHDARRVYLILEYAPRGELYKELQKSEKLDEQRTATIIEEVADALTYCHDKKVIHRDIKPENLLL
GFRGEVKIADFGWSVHTPLPERKTMCGTLDYLPPEMIEGRTYDEKVDLWCIGVLCYELLVGYPPFESASH

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SETYRRILKVDVRFPLSMPLGARDLISRLLRYQPLERLPLAQILKHPWVQAHSRRVLPPCAQMAS

>gi|4507275|ref|NP_003591.1| serine/threonine kinase 6; serine/threonine protein kinase-6; aurora-A [Homo sapiens]
MDRSKENCISGPVKATAPVGGPKRVLVTQQIPCQNPLPVNSGQAQRVLCPSNSSQRVPLQAQKLVSSHKP
VQNQKQKQLQATSVPHPVSRPLNNTQKSKQPLPSAPENNPEEELASKQKNEESKKRQWALEDFEIGRPLG
KGKFGNVYLAREKQSKFILALKVLFKAQLEKAGVEHQLRREVEIQSHLRHPNILRLYGYFHDATRVYLIL
EYAPLGTVYRELQKLSKFDEQRTATYITELANALSYCHSKRVIHRDIKPENLLLGSAGELKIADFGWSVH
APSSRRTTLCGTLDYLPPEMIEGRMHDEKVDLWSLGVLCYEFLVGKPPFEANTYQETYKRISRVEFTFPD
FVTEGARDLISRLLKHNPSQRPMLREVLEHPWITANSSKPSNCQNKESASKQS

TABLE1

MAX symbol	MAX name aliases	MAX RefSeq_NA or GI_NA	MAX GI_AA	MAX NAME	MAX DESCRIPTION	MAX PROTEIN LENGTH	MODIFIER GI_AA
MBTPS2	S2P, S2P protein,membrane-bound transcription factor protease, site 2	NM_015884.1	7706693	membrane-bound transcription factor protease, site 2	metalloendopeptidase	519	17556258
PAPSS1	SK1, ATPSK1, 3-prime-phosphoadenosine 5-prime-phosphosulfate synthase 1,3'-phosphoadenosine 5'-phosphosulfate synthase 1	NM_005443.2	20127475	3'-phosphoadenosine 5'-phosphosulfate synthase 1	kinase; kinase; sulfate adenylyltransferase (ATP); sulfate adenylyltransferase (ATP)	624	17542422

TABLE1

MAX symbol	MAX name aliases	MAX RefSeq_NA or GI_NA	MAX GI_AA	MAX NAME	MAX DESCRIPTION	MAX PROTEIN LENGTH	MODIFIER GI_AA
PAPSS2	SK2, ATPSK2, 3-prime-phosphoadenosine 5-prime-phosphosulfate synthase 2,3'-phosphoadenosine 5'-phosphosulfate synthase 2	NM_004670.1 XM_005865.2	4758880	3'-phosphoadenosine 5'-phosphosulfate synthase 2	kinase	614	17542422
STK12	AUR1,AIK2,AIM1,ARK2,IPL1,AIM-1,STK-1,serine/threonine kinase 12	NM_004217.1	4759178	serine/threonine kinase 12	protein kinase; protein kinase; protein serine/threonine kinase; protein serine/threonine kinase	344	17505246
STK13	AUR3,AIE2,AIK3,AIE1_m,serine/threonine kinase 13 (aurora/IPL1-like)	NM_003160.1 XM_030228.2	4507273	serine/threonine kinase 13 (aurora/IPL1-like)	protein kinase; protein serine/threonine kinase	275	17505246

60401534 .080604

TABLE1

MAX symbol	MAX name aliases	MAX RefSeq_NA or GI_NA	MAX GI_AA	MAX NAME	MAX DESCRIPTION	MAX PROTEIN LENGTH	MODIFIER GI_AA
STK6	AIK, BTAK, STK15, aurora-A, serine/threonine protein kinase-6,serine/threonine kinase 6	NM_003158 NM_003600.1	4507275	serine/threonine kinase 6	protein kinase; protein serine/threonine kinase	403	17505246